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Research Article

RESEARCH ARTICLE

Functional Dissociation of Group III Metabotropic Glutamate Receptors Revealed by Direct Comparison between the Behavioral Profiles of Knockout Mouse Lines

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Abstract

Background: Group III metabotropic glutamate receptors (mGlu4, mGlu7, mGlu8) display differential brain distribution, which suggests different behavioral functions. However, comparison across the available animal studies remains methodologically hazardous and controversial. The present report directly compares knockouts for each group III receptor subtype using a single behavioral test battery and multivariate analysis.
Methods: The behavioral phenotypes of C57BL/6J mice lacking mGlu4, mGlu7, or mGlu8 and their respective littermates were examined using a multimetric test battery, which included elements of neuromotor performance, exploratory behavior, and learning and memory. Multivariate statistical methods were used to identify subtype-specific behavioral profiles and variables that distinguished between these mouse lines.
Results: It generally appears that mGlu7 plays a significant role in hippocampus-dependent spatial learning and in some fear-related behaviors, whereas mGlu4 is most clearly involved in startle and motivational processes. Excepting its influence on body weight, the effect of mGlu8 deletion on behavior appears more subtle than that of the other group III receptors. These receptors have been proposed as potential drug targets for a variety of psychopathological conditions.
Conclusion: On the basis of these controlled comparisons, we presently conclude that the different group III receptors indeed have quite distinct behavioral functions.

Keywords: Metabotropic glutamate receptors, knockout mice, behavioral phenotyping, behavioral test battery

Introduction

Metabotropic glutamate (mGlu) receptors, belonging to the G-protein–coupled receptor family, are thought to mediate slow, modulatory signals, whereas ionotropic glutamate receptors mediate fast synaptic responses (Pin and Duvoisin, 1995; Schoepp, 2001). The 8 mGlu receptor subtypes identified so far (mGlu1–mGlu8) have been segregated into 3 receptor groups, according to sequence homology, pharmacology, and signal transduction mechanisms (Pin and Duvoisin, 1995). Many authors emphasized the putative importance of these various mGlu receptors in brain physiology and pathophysiology but deplored their largely unexploited potential as therapeutic drug targets (Swanson et al., 2005; Récasens et al., 2007; Niswender and Conn, 2010).

Differential brain distribution in presynaptic receptors belonging to group III (mGlu4, mGlu6, mGlu7, mGlu8) suggests functional dissociation between these receptors (Wu et al., 1998; Dobi et al., 2013). The functions of mGlu6 shall not be further

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discussed here, because of its restriction to the inner layer of the retina (Nakajima et al., 1993). In contrast, mGlu7 appears to be mainly expressed in telencephalic areas (neocortex, hippocampus, etc.), whereas mGlu4 and -8 are relatively more prominent in lower brain areas (see Table 1 for an overview of expression levels of group III receptors throughout the rodent brain). Consistent with its high expression in cerebellar granule cells (Tanabe et al., 1993; Kinoshita et al., 1996; Mateos et al., 1998), mGlu4 has been implemented in motor learning (Pekhletski et al., 1996; Davis et al., 2012). The prominent telencephalic distribution of mGlu7 (Kinoshita et al., 1998) is definitely consistent with its putative role in learning and memory, anxiety, and depression-related behaviors (Cryan et al., 2003; Callaerts-Vegh et al., 2006; Palucha et al., 2007; Fendt et al., 2013). Finally, mGlu8 has been found mainly in olfactory bulb, olfactory tubercle, and mammillary bodies (Duvoisin et al., 1995), but reports about its precise behavioral function remain inconclusive (Gerlai et al., 2002; Duvoisin et al., 2005; Fendt et al., 2010; Davis et al., 2013).

Unfortunately, none of the previously published reports directly compared the functional features of the 3 group III receptor subtypes. Each receptor subtype has been examined separately in these reports, using different background strains, protocols, variables, etc. Comparing behavioral outcomes between studies, models, and protocols is notoriously difficult and controversial (Crabbe et al., 1999). Therefore, the goal of the present study was to compare directly between mGlu4, mGlu7, and mGlu8 knockout mice, backcrossed to the same genetic background, and subjected to the same multimetric test battery (Goddyn et al., 2006). The behavioral test battery included a broad range of neuromotor, exploratory, and cognitive tasks. Relevant multivariate statistical techniques were used to compare the different behavioral profiles and identify discriminating variables (Leighty et al., 2004).

Methods

Animals

Female mGlu4, mGlu7, and mGlu8 knockout mice ($mGlu4^{-/-}$, $mGlu7^{-/-}$, $mGlu8^{-/-}$) were generated and backcrossed to C57BL/6J background as described previously (Duvoisin et al., 2005). Age-matched mGlu4, mGlu7, and mGlu8 wild-type littermates were used as controls ($mGlu4^{+/+}$, $mGlu7^{+/+}$, $mGlu8^{+/+}$), and all genotypes were confirmed using PCR-based methods as described. All in all, 108 mice were examined ($mGlu4^{-/-}$ n = 22, $mGlu7^{-/-}$ n = 14, $mGlu8^{-/-}$ n = 17, wild-type mice n = 55; wild-type mice were pooled,

Table 1. Brain Distribution of mGlu4, -7, and -8 in Laboratory Rodents

					Hippocampus							
Subtype		Olfactory Bulb	Neocortex	Striatum	Overall	CA1	CA3	DG	Thalamus	Amygdala	Cerebellum	Brainstem
mGlu4	IH:		-[a] +/-[c,k]	+[c, k]	+/-[c]	-[r] +[a]	-[r] +[a]	+[a,r]	-[a] +[c]	+/-[c]	+++[a, c,f]	
	IS:	+++[t]	-[u] - -[m]	-[u] ++[l]	-[u]		-[t]	-[t]	+/-[u] +++[l, t]		+++[m,t, u]	
mGlu7	IH:	+[g, j,n] ++[v]	++[g]	-[g]	++[s]	++[g,r]	++[g] +++[r]	++[r] +++[g]	+/-[g]	+[g]	- -[g]	
	IS:	++[h, j, n]	++[h,j, m, n] +++[b]	+[h] ++[j,] +++[b, l]	++[h, n] +++[b]	++[j]	+++[j]	+++[h,j]	+[j,h] ++[l, n] +++[b]	+[h]	-[h] +/-[j]	+[h]
mGlu8	IH:	++[v]				-[r] +[e]	+[r] ++[e]	+[r] ++[e]		+[o]		+[p]
	IS:	++[b] +++[d,q]	+[b,q]	++[t]	- -[d] +[q]				+[b, l] +++[q]	+[b]	- -[d] +[q]	

Intensity of immunoreactivity: +++ most intense, ++ intense, + moderate, +/- moderate to weak, - weak, - - negative; IH: immunohistochemistry; IS: in situ hybridization.

[a(*)] Bradley et al. (1999).
[b]Corti et al. (1998).
[c(*)] Corti et al. (2002).
[d*]Duvoisin et al. (1995).
[e]Ferraguti et al. (2005).
[f*]Kinoshita et al. (1996).
[g(*)] Kinoshita et al. (1998).
[h]Kinzie et al. (1995).
[i]Kinzie et al.(1997).
[j]Kosinski et al. (1999).
[k]Kuramoto et al. (2007).
[l]Messenger et al. (2002).
[m]Ohishi et al. (1995).
[n]Okamoto et al. (1994).
[o]Palazzo et al. (2011).
[p]Pamidimukkala et al. (2002),
[q]Saugstad et al. (1997).
[r]Shigemoto et al. (1997).
[s]Somogyi et al. (2003).
[t]Tanabe et al. (1993).
[u]Testa et al. (1994.
[v]Wada et al. (1998).
* data in mice; (*) similar data in rat and mice.

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since multivariance analysis revealed no statistical difference between the 3 wild-type groups). Mice were bred at the Janssen Pharmaceutica facilities (Beerse, Belgium) and transferred to Leuven University at the age of approximately 12 weeks. Mixed genotype groups were kept in standard animal cages in temperature- and humidity-controlled rooms (12-h-light/-dark cycle, 22°C). Food and water were available ad libitum, unless stated otherwise. Behavioral experiments were conducted during the light phase of the activity cycle. All protocols have been reviewed and approved by the Animal Experiments Committee of KU Leuven in accordance with the European Community Council Directive (86/609/EEC).

Behavioral Test Battery

Neuromotor Performance and Prepulse Inhibition

Mice were first tested in neuromotor tests, because alterations in general cage activity, motor coordination, and grip strength could confound performance in other behavioral tasks. To measure circadian cage activity, mice were placed individually in standard transparent cages (26.7 cm × 20.7 cm) located between 3 infrared photo beams. For 23 hours, activity was measured by a laboratory-built activity logger and expressed as beam crossings for each 30-minute interval. Grip strength was measured using a T-shaped bar connected to a digital dynamometer (Ugo Basile, Comerio, Italy). Mice were placed on the apparatus so that they spontaneously grabbed the bar and were gently pulled backwards until they released the bar. Maximal strength (in mN) was recorded 10 times and averaged per animal.

Motor coordination and equilibrium were tested on an accelerating rotarod (MED Associates Inc., St. Albans, VT). Mice were first trained at constant speed (4 rounds/min, 2 minutes) before starting with 4 test trials (inter-trial interval, 10 minutes). During these trials, mice had to balance on the rotating rod that accelerated from 4 to 40 rounds/min in 5 minutes. Time until they dropped from the rod was recorded up to a maximum of 5 minutes.

Prepulse inhibition (PPI) was assessed in a sound attenuating cubicle with a load cell platform (MED Associates Inc) as described before. Mice were placed in a small animal holder that restricted movement and placed on the platform. After a 5-minute acclimation period, 5 initial startle pulses were delivered (115 dB, 5 kHz, 40 ms). Subsequently, 10 trial blocks were presented. Each block consisted of startle pulse alone (SP: 115 dB; 5 kHz; 40 ms), 3 SPs preceded by prepulses (SPPP; 70, 75, 80 dB; 5 kHz, 20 ms), and 3 prepulses (PP) alone. Within each block, trial types (SP, SPPP, or PP) were administered at random with an inter-trial interval of on average 15 seconds. Startle reactivity was recorded during 200 ms from stimulus onset in all SP trials (SP or SPPP) and PP trials. A 200-ms interval just before stimulus onset was recorded as baseline measurement ("null" interval). During the trial, a constant background noise was delivered (white noise, 50 dB). Acoustic startle response (ASR), that is, reactivity to the SP alone, and percentage PPI were recorded. PPI was calculated for each PP intensity from peak values according to the following formula: %PPI = [1 – (startle peak at SP after PP) / (ASR)]*100.

Exploration

Open field (OF) and social exploration (SE) were examined using a 50 cm × 50 cm arena. Animals were dark adapted for 30 minutes and placed in a corner of the arena. After 1 minute of exploration, movements of the mice were recorded for 10 minutes using EthoVision video tracking equipment and software (Noldus, Wageningen, The Netherlands). Total path length, rearing frequency, corner crossings, center entries, and percentage path length in the center were recorded. In the SE test, 2 female mice were placed in a centrally located cage enabling visual, olfactory, and limited physical contact. In the elevated plus maze test (EPM), the arena consisted of a plus-shaped maze with 2 open and 2 closed arms (5 cm wide). Mice were placed at the center of the maze and were allowed to explore freely for 10 minutes (after 1 minute of adaptation). Five infrared beams (4 for arm entries and 1 for open-arm dwell), connected to a computerized activity logger, recorded exploratory activity. Total number of arm entries (ie, beam crossings in open and closed arms), percentage of open arm entries, and open-arm dwell (ie, percentage of time per minute spent in the open arms) were measured.

Learning and Memory

Learning and memory abilities were examined in 3 tasks. Single trial passive avoidance (PA) learning was examined in a step-through box with a shock grid. The box consisted of an illuminated compartment and a dark compartment separated by a guillotine door. After a 30-minute dark adaptation period, animals were placed in the light part, and after 5 seconds the sliding door to the dark compartment was opened. Latency to enter the dark compartment was measured. On entry of the dark compartment, the door was closed and a 2-second foot shock (0.2 mA) was delivered by a constant current shocker (MED Associates Inc). Twenty-four hours later, mice were again placed in the light box, and latency to enter the dark compartment was measured.

Spatial learning capacity was examined in the standard hidden platform version of the Morris water maze (MWM) as previously described (Goddyn et al., 2006). Briefly, a circular pool (diameter 150 cm, depth 32.5 cm) filled with water (26°C, opacified with nonoxic white paint) to a depth of 16 cm, contained a circular hidden platform (15 cm diameter). Mice were trained for 10 days (4 trials/d; ITI of 15 minutes) to find the hidden escape platform, starting randomly from each of 4 starting positions. Mice that failed to find the hidden platform within 2 minutes were gently guided to the platform, where they remained for 15 seconds before being returned to their home cage. Escape latency, path length, swim velocity, and time spent near the wall (thigmothaxis) were recorded with EthoVision video tracking equipment and software (Noldus, Wageningen, The Netherlands). Probe trials were conducted after 5 training days. During these probe trials, the platform was removed from the pool, and the search pattern of the mice was recorded for 100 seconds. Time spent in each quadrant, path length, latency of first entrance in the target quadrant, and mean distance to the former platform location were calculated.

Finally, contextual fear conditioning (CFC) was based on a protocol used by Paradee et al. (1999). On the first day, animals were placed in the StartFear cage (Panlab, Spain) with black walls and a grid floor. Animals were allowed to acclimate to the box for 5 minutes and were then returned to their home cage. On the second day, after 2 minutes of exploration (baseline), a 30-second tone was delivered co-terminating with a 2-second shock (0.3 mA). After another minute of exploration, another tone-shock pairing was delivered followed by 1 minute of exploration. Twenty-four hours later, animals were returned to the testing chamber for 5 minutes of exploration (context trial). After 90 minutes in their home cage, animals were placed in a white paper box inside the StartFear cage (different context) for 6 minutes. After the 3 minutes (preCS trial), the tone was delivered for 3 minutes (CS trial). During each trial, freezing behavior was recorded by a sensitive Weight Transducer system (Panlab, Spain). The percentage of freezing was calculated per trial.

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Statistics

Data are presented as mean and SEM. Differences between mean values were determined using both parametric (1-way ANOVA with Tukey tests for posthoc comparison) and nonparametric (Kruskal-Wallis) tests. Within-subject trials were compared using repeated-measures ANOVA (RM-ANOVA) with Greenhouse-Geisser correction in case of sphericity violation. Outliers were defined by total beam crossings in the circadian activity task. Mice with values lower or higher than 1.5 times the interquartile range below or above the 25th or 75th percentile, respectively, were excluded from all analyses.

Correlation analysis was used to examine the relationships between the different behavioral measures. Pearson correlations were calculated for within and between task variables. The complete list of behavioral variables is provided in Table 2. Discriminant function analysis (direct and stepwise DFA) was used to examine which variables contributed significantly to differences between genotypes. Therefore, only knockout mice were included in this analysis. In direct DFA, all variables are included at once and one can examine the significance of discriminability between genotypes. The stepwise forward approach begins with no variables in the model and, based on statistical criteria, variables (one at the time) that contribute significantly to differences between the groups are selected. Direct entry and stepwise DFA were executed using: (1) all variables, (2) only neuromotor variables, (3) only exploratory variables, and (4) only cognitive measures. All analyses were conducted using SPSS 19.0 statistical package (SPSS Inc, Chicago IL) at $\alpha = 0.05$.

Results

Neuromotor Performance and PPI

Cage activity was measured by the number of beam crossings. Boxplots of total beam crossings identified 2 mGlu8$^{-/-}$ mice as outliers, which were removed from further analysis. One-way RM-ANOVA (Greenhouse-Geisser correction) with genotype as between-subjects and time as within-subjects variable revealed a significant main effect of time [$F(14,1417) = 66.54$; $p<.001$], and a significant time by genotype interaction [$F(42;1417) = 1.68$; $P = .005$], but no significant main effect of genotype [$F(3,102) = 1.02$] on circadian activity. Posthoc comparisons indicated that, at the first hour of the test, mGlu7$^{-/-}$ mice made fewer beam crossings than wild-type animals (16.30: $P = .004$, 17.00: $P = .014$). At the start of the second light phase, mGlu4$^{-/-}$ mice made significantly fewer beam crossings (Figure 1).

No significant differences were observed between subtype-specific knockout and wild-type mice in the grip strength and rotarod tasks [$F(3,102) = 2.56$; $F(3,102) = 0.58$]. One-way ANOVA on weight revealed a significant main effect [$F(3,102) = 15.14$; $P < .001$]. Posthoc pairwise comparisons showed that mGlu7$^{-/-}$ mice weighed less ($M = 19.45$) than all other subtype-specific knockout groups and wild-type mice (all P-values $<.001$). On the other hand, mGlu8$^{-/-}$ ($M = 25.07$) weighed somewhat more than wild-type mice ($M = 23.03$; $P = .016$).

To assess afferent functions and sensorimotor gating, we recorded ASR and PPI in the different genotypes (recordings in one mGlu8$^{-/-}$ animal were discarded for technical reasons). One-way ANOVA examined the effect of genotype on ASR. A significant effect of genotype [$F(3,101) = 5.36$; $P = .002$] indicated that mGlu4$^{-/-}$ mice showed less ASR than all other subtypes (mGlu7$^{-/-}$: $P = .011$, mGlu8$^{-/-}$: $P = .017$) and wild-type animals ($P = .003$; Figure 2A). Repeated-measures ANOVA on percentage PPI showed an effect of genotype [$F(3,101) = 5.29$; $P < .02$] and pulse intensity [$F(2,202) = 12.405$; $P < .001$]. Posthoc tests indicated that percentage PPI was significantly lower in mGlu4$^{-/-}$ mice than in mGlu7$^{-/-}$ and wild-type animals (Figure 2B). Because of a possible influence of the initial difference in ASR on percentage PPI (%PPI calculation is partially based on ASR data), correlation analysis and RM-ANOVA with ASR as covariate were executed.

Table 2. List of Behavioral Variables Recorded in mGlu4, mGlu7, and mGlu8 Knockout and Wild-Type Mice

Behavioral Task	Variables	Abbreviation
Cage activity	Total beam crossings	A-TOT
	Beam crossings during first half hour	A_30
Grip	Mean	GRIP
Rotarod	Total time on rod	ROT
PPI task	Startle	ASR
	%PPI with prepulse of 75dB	PPI_75
Open field	Path length	OF_PL
	Rearing	OF_R
	Percentage path length in centre	OF_PLc
	Latency of first centre entry	OF_LAT
Social exploration	Path length	SE_PL
	Rearing	SE_R
	Percentage path length in centre	SE_PLc
	Latency of first centre entry	SE_LAT
Elevated plus maze	Total beam crossings	EP_TOT
	Open arm dwell	EP_OAD
Passive avoidance	Test-training latency	PA
Morris water maze	Total path length day 1	MW_T1PL
	Difference in PL between first and fifth day	MW_PL
	Average velocity week 1	MW_VEL1
	Time in target probe 1	MW_TAR1
Contextual fear conditioning	Percentage freezing shock	CF_SH
	Percentage freezing context	CF_CXT
	Percentage freezing cue	CF_CUE

Abbreviations:

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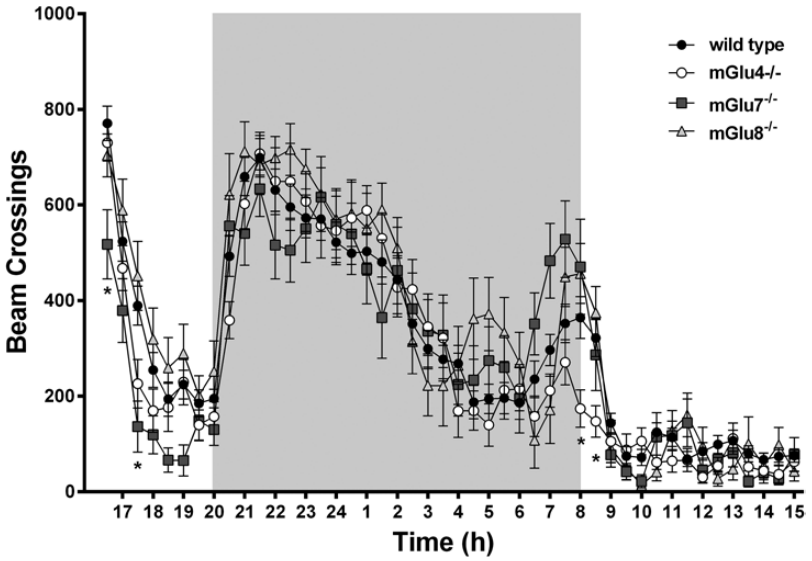

Figure 1. Circadian activity in wild-type, mGlu4$^{-/-}$, mGlu7$^{-/-}$, and mGlu8$^{-/-}$ mice (dark phase from 8 PM to 8 AM, grey block). No gross alterations in behavioral activity could be observed. However, during the first hour, mGlu7-/- mice (dark grey squares) were less active than wild-type animals (filled circles), while during the start of the second light phase mGlu4$^{-/-}$ mice (open circles) were less active. Data are represented as mean ± SEM. Asterisk indicates significant difference from the wild-type group: *$P < .05$.

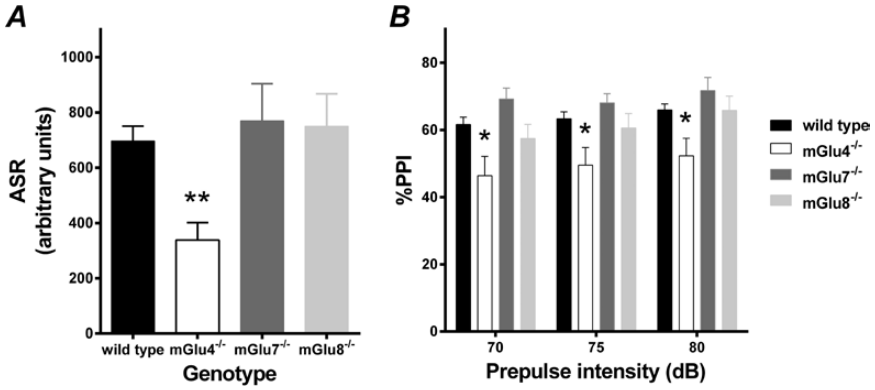

Figure 2. ASR and PPI measures in wild-type and mGlu knockout mice. (A) A clear decrease in acoustic startle reactivity (ASR) is observed in mGlu4$^{-/-}$ mice (white bar). (B) For all 3 prepulse intensities, % PPI (prepulse inhibition) was lower in mGlu4$^{-/-}$ mice (white bars) than in wild-type animals (black bars). Data are represented as mean (A) and estimated marginal mean (B) ± SEM. Asterisks indicate significant difference with wild-type group, *$P < .05$, **$P < .01$.

Correlation analyses demonstrated significant linear relationships between ASR and percentage PPI in mGlu4$^{-/-}$ mice (70dB: $r = 0.52$, $P = .013$; 75B: $r = 0.49$; $P = .021$; 80dB: $r = 0.54$; $P = .01$) but not in the other groups of animals. In addition, the main effect of genotype on percentage PPI was still significant in repeated-measures ANCOVA with ASR as covariate [$F(3,100) = 4.16$; $P = .008$]. mGlu4$^{-/-}$ mice had a significantly lower percentage PPI than mGlu7$^{-/-}$ mice ($P = .01$) and wild-type mice ($P = .02$). These 2 additional analyses indicate that the decreased percentage PPI in mGlu4$^{-/-}$ mice might reflect a real decrease rather than only a decreased ASR.

Exploration

Anxiety-related behavior can be assessed in alterations of exploration pattern in OF, SE, and EPM. In these tests, no gross behavioral differences were observed between knockout and wild-type mice (Table 3). Notably, mGlu7$^{-/-}$ mice showed less rearing behavior in both the OF [$F(3,102) = 3.48$; $P = .019$] and SE [$F(3,102) = 6.02$; $P = .001$]. While mGlu4$^{-/-}$ mice had a shorter path length in the SE [$F(3,102) = 3.79$; $P = .013$].

Learning and Memory

Learning and memory capacity was investigated in PA, MWM, and CFC, 3 well-known paradigms to assess cognitive abilities in rodents. In PA learning, no effect of genotype could be observed on the latency to enter the dark compartment on the second day (wild-type: 195.15 ± 15.51 seconds; mGlu4$^{-/-}$: 166.55 ± 27.72 seconds; mGlu7$^{-/-}$: 181.21 ± 31.89 seconds; mGlu8$^{-/-}$:224.47 ± 26.16 seconds).

In the MWM, all mice learned to locate the hidden platform during the first week of acquisition training, reflected by a prominent decrease in escape latency and path length (Latency: $F(2.82;284.87) = 81.89$; $P < .001$; path length: $F(3.04;306.71) = 96.66$; $P < .001$) (Figure 3). However, RM-ANOVA on path length revealed a significant main effect of genotype (with no significant interaction), and additional posthoc comparisons indicated a significant delay in acquiring the exact platform location in mGlu7$^{-/-}$ mice [$F(3,101) = 4.063$; $P = .009$; posthoc: mGlu7$^{-/-}$ vs wild-type: $P = .044$, mGlu7$^{-/-}$ vs mGlu4$^{-/-}$: $P = .005$] (Figure 3B). mGlu4$^{-/-}$ mice displayed a significantly shorter latency to reach the target platform [$F(3,101) = 3.47$; $P = .019$; posthoc: mGlu4$^{-/-}$ vs wild-type: $P = .045$, mGlu4$^{-/-}$ vs mGlu7$^{-/-}$: $P = .02$]. Correspondingly, mGlu4$^{-/-}$

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Table 3. Exploratory Behavior in mGlu4, mGlu7, and mGlu8 Knockout and Wild-Type Mice

Task	Variable	Wild-Type	mGlu4$^{-/-}$	mGlu7$^{-/-}$	mGlu8$^{-/-}$
OF	Path length (in cm)	3969(129)	3567(136)	3823(251)	4064(209)
	Rearing	32.1(2.9)	40(6)	16.2(2.3)	29.7(2.6)
	% path length centre	25.4(0.9)	25.4(1.4)	23.8(2.2)	23.0(1.5)
SE	Path length (in cm)	4093.36(125)	3349(224)*	3815(298)	4225(190)
	Rearing	37.6(2.3)	32(4)	21(4)**	45.4(2.7)
	% path length centre	49.3(2.4)	44(4)	40(6)	52.1(2.4)
EPM	Total beam crossings	135(3)	135(6)	134(8)	143(6)
	Open arm dwell %	18.7(1.0)	21.9(1.3)	19.9(1.5)	17.3(2.5)

Abbreviations: EPM, elevated plus maze; OF, open field; SE, social exploration. Data are means (SEM). Asterisks indicate significant difference with wild-type animals: *P < .05; **P < .01.

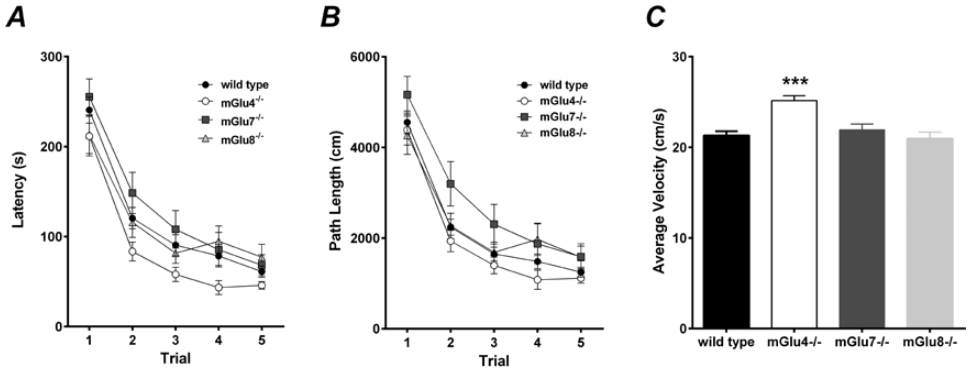

Figure 3. Comparison of spatial learning between wild-type and mGlu group III knockout mice in the Morris water maze during the first acquisition week. (A) Escape latency data demonstrate that all mice learned to locate the platform by the end of week 1. However, mGlu4$^{-/-}$(open circles) reached the platform significantly faster than wild-type (black dots) and mGlu7$^{-/-}$ (dark grey squares) animals. (B) mGlu7$^{-/-}$ mice (dark grey squares) swam a longer distance to reach the platform compared to wild-type (filled circles) and mGlu4$^{-/-}$ (open circles) mice. Path length is considered to be a more cognitive measure. (C) Velocity data show a clear increase in swimming speed in mGlu4$^{-/-}$ mice (white bar). Data are presented as mean ± SEM, ***P < .001 compared with wild-type animals.

mice showed a significantly increased swimming velocity [F(3,101) = 8.52; P < .001] (Figure 3C).

During the second week of acquisition training, no differences could be observed between genotypes on path length and latency. However, mGlu4$^{-/-}$ mice still swam faster than wild-type animals [F(3,101) = 2.78; P = .045; posthoc: mGlu4$^{-/-}$ vs wild-type: P = .048].

At the end of each trial week, a probe trial was performed to measure the spatial accuracy of the mice. Already in the first probe trial, all mice showed a clear preference for the target quadrant in comparison with the other quadrants, indicated by a main effect of quadrant [F(2,212) = 46.62; P < .001] with no significant interaction [F(6,212) = 0.958] or main effect of genotype [F(3,101) = 1.22]. Similar to acquisition trials, a main effect of velocity was observed [F(3,101) = 4.47; P = .005]. More specifically, mGlu4$^{-/-}$ mice swam significantly faster than wild-type mice (P = .003) and mGlu8$^{-/-}$ mice (P = .044).

In CFC, mice had to learn the association between a cue (tone) and context (test cage), and an aversive event (shock). RM-ANOVA revealed a main effect of genotype [F(3,102) = 4.08; P = .009] and trial [F(3,325) = 179.41; P < .001] but no significant interaction-effect [F(10,325) = 0.94]. In general, mGlu7$^{-/-}$ mice showed an overall decrease in freezing behavior (mGlu7$^{-/-}$ vs mGlu4$^{-/-}$: P = .005) (Figure 4).

Correlations between Behavioral Variables

Correlation analyses revealed intra- and inter-task correlations. Overall, high correlations were found between neuromotor measures, between exploratory measures, and between cognitive measures, strengthening the face-validity of grouping these tasks (as previously shown in Caeyenberghs et al., 2006).

Analyzing receptor-deficient and wild-type animals separately, different correlation patterns were observed. First, no significant correlations with weight were observed in mGlu7 and mGlu8 knockout mice. In mGlu4$^{-/-}$ mice, a significant negative correlation between weight and rearing in the OF (OF_R) was observed (r = -0.43; P = .044). In wild-type animals, weight significantly correlates positively with grip strength and inversely with freezing to context in the contextual fear task.

Intra- and inter-task correlations of OF and SE are strongest in wild-type and mGlu4 knockout mice. Data of mGlu7 knockout mice demonstrate only intra-task correlation of SE, while in mGlu8 knockout mice, the pattern is more scattered. In cognitive tasks, wild-type and mGlu7 knockout mice displayed high intra-task correlations in the CFC task. In mGlu4 and mGlu8 knockout mice, only shock and context freezing data correlated significantly. Wild-type, mGlu4, and mGlu8 knockout mice show intra-task correlations between MWM performance variables, whereas none of these measures correlated significantly in mGlu7 knockout mice. A highly significant negative correlation (r = -0.47; P = .005) between velocity in week 1 (MW_VEL1) and path length during the first trial block was observed in mGlu4$^{-/-}$ mice. In mGlu7$^{-/-}$ mice, high positive correlations were observed between intra-task measures in the CFC task, while freezing to tone correlated negatively with total beam crossings in the cage activity (r = -0.67; P = .009) and EPM task (r = -0.56; P = .039).

DFA

Results of DFA are summarized in Table 4. The 3 group III receptor subgroups could be discriminated when all behavioral measures were included in the model (direct entry method). Using

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the stepwise method, body weight, OF_R, swimming velocity in MWM (MW_VEL1), and freezing to tone during CFC provided maximal discriminability (84.3% of cases were classified correctly based on these measures). The DFA resulted in 2 significant canonical functions accounting for 71.3% (Eigenvalue = 1.83) and 28.7% of variance (Eigenvalue = 0.74), respectively (Figure 5). Weight was most strongly correlated with the first function, while OF_R, CFC_CS, and MWM_VEL1 were more strongly correlated with the second function.

The 3 groups could not be discriminated when all 6 neuromotor variables were entered simultaneously, but maximal dissociation was achieved with mean grip strength and ASR using the stepwise method. When all exploratory variables were included in the direct entry model, the 3 receptor-deficient groups could be discriminated. The stepwise method showed that OF_R and SE task (SE_R) were important discriminators.

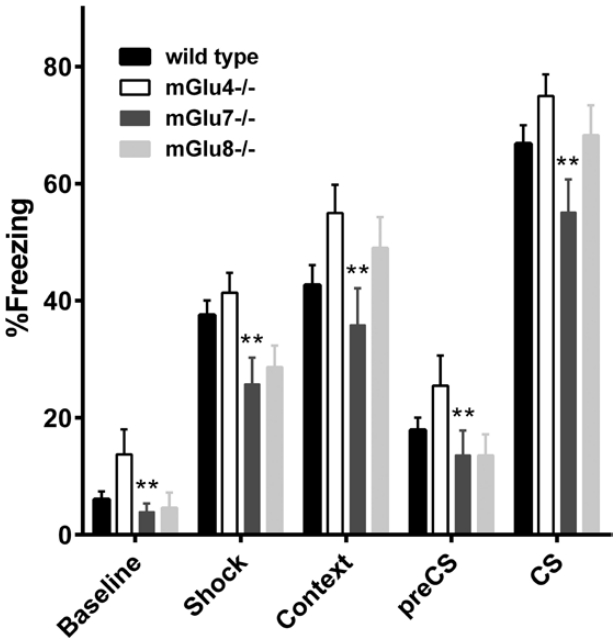

Figure 4. Contextual fear conditioning in mGlu group III knockout and wild-type mice. All mice learned to associate the context (increased freezing in "context" phase) and tone (increased freezing in CS phase) to the shock. Over all phases, mGlu7$^{-/-}$ mice (dark grey bars) display less freezing. ** $P < .01$ compared with mGlu4$^{-/-}$ mice. Data are presented as mean ± SEM.

Lastly, the stepwise method revealed significant discriminability between the 3 genotypes using cognitive measures. Velocity and difference in path length between first and last day of MWM performance provided maximal discriminability.

Discussion

Subtype-specific knockout mice have been generated, amongst other things, to investigate the behavioral functions of group III metabotropic receptors and their potential as drug targets for the treatment of neuropathological and mood disorders (Cryan et al., 2003; Goddyn et al., 2008; Fendt et al., 2013; Iscru et al., 2013). However, few of these studies actually analyzed a broader range of behaviors, and none of them compared different group III knockouts directly in a single behavioral battery. Therefore, we tested mGlu4, mGlu7, and mGlu8 knockout mice simultaneously on multiple behavioral tasks, and multivariate statistical techniques were used to uncover subtype-specific behavioral profiles. Their differential brain distribution suggested already that mGlu4, mGlu7, and mGlu8 receptors may serve different behavioral functions, which is definitely confirmed by the present study showing significant functional dissociation between subtype-deficient mice in PPI, MWM, and CFC. Notably, mGlu8$^{-/-}$ mice did not show any behavioral alteration in comparison with wild-type animals throughout the entire test battery. They were significantly heavier than the other mice, and indeed substantial weight gain has been reported previously in mGlu8 knockout mice (Duvoisin et al., 2005; Davis et al., 2013).

In the acoustic response tasks, mGlu4$^{-/-}$ mice showed decreased ASR and PPI, whereas these measures were unaltered in mGlu7 and mGlu8 knockout mice. Notably, covariance analysis indicated that ASR decrease is not responsible for the subsequently observed decrease in percentage PPI. This is in accordance with a study by Paylor and Crawley (1997), which demonstrated that sensorimotor gating (as measured by percentage PPI) and acoustic startle are in fact independent functions. The mGlu4 receptor appears to be expressed in glutamatergic and GABAergic synapses in brain structures that are involved in acoustic startle functions. Startle reactivity is mainly a brainstem reflex that is modulated by cortico-striato-pallido-pontine circuitry (Swerdlow et al., 2000). Weber et al. (2002) hypothesized that glutamate release from auditory afferents in nucleus reticularis pontis caudalis may be inhibited by presynaptically located group III mGlu receptors (still, the actual presence of group III receptors in this structure remains to be demonstrated). Even

Table 4. DFA on Behavioral Variables of mGlu4, mGlu7, and mGlu8 Knockout and Wild-Type Mice

		Stepwise Forward Method	
Included Variables	Direct Entry Method	Significance	Variables Retained
All variables	$P = .030$	$P < .001$	Weight MWM_VEL1 CFC_CUE OF_R
Neuromotor	N.S.	$P = .002$	GRIP ASR
Exploratory	$P = .042$	$P < .001$	OF_R SE_R
Cognitive	N.S.	$P < .001$	MWM_VEL1 MWM_PL

Abbreviations: ASR, acoustic startle response; CFC_CUE, contextual fear conditioning, percentage freezing cue; MWM_PL: Morris water maze, difference in path length between day 1 and day 5; MWM_VEL1, Morris water maze average velocity week 1; N.S., not significant; OF_R, open field rearing; SE_R: social exploration rearing.

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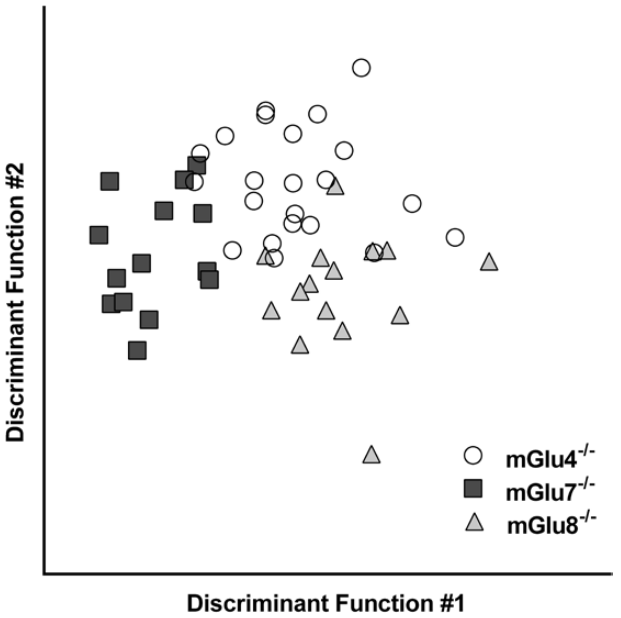

Figure 5. Scatterplot using discriminant scores clearly illustrates the distinct behavioral profiles of the 3 mGlu knockout groups (mGlu4$^{-/-}$: open circles; mGlu7$^{-/-}$: dark grey squares; mGlu8$^{-/-}$: light grey triangles). This plot depicts discriminant scores of the 2 discriminant functions derived from behavioral and body weight measures (Table 2). Body weight was more strongly correlated with discriminant function 1, while open filed rearing (OF_R), swimming velocity in Morris water maze (MWM_VEL1), and freezing to tone in the contextual fear conditioning paradigm (CFC_CS) were more strongly correlated with function 2.

more significantly, mGlu4 receptors are expressed in basal ganglia and hippocampus, which are both intricately involved in startle-related functions (Shoemaker et al., 2005). Alterations in presynaptic inhibition of GABA and/or glutamate release in these key structures could definitely account for the decrease in startle reactivity (and even PPI) presently observed in mGlu4$^{-/-}$ mice. Similar results have been observed after NMDA infusion in rat ventral hippocampus (Zhang, 2001). Since mGlu4 receptors are known to inhibit presynaptic glutamate release in hippocampus (Phillips et al., n.d.), mGlu4 deletion may have altered ASR and PPI by affecting hippocampal NMDA receptor activity. It should also be noted that disrupted ASR and PPI have been found in a variety of neuropsychiatric disorders, such as schizophrenia and Huntington disease (Swerdlow et al., 1995, 2008). Swanson et al. (2005) specifically mentioned mGlu7 and mGlu8 as promising drug targets for anxiety and depression disorders. In several studies, mGlu7 knockout mice showed an anxiolytic-like phenotype (Cryan et al., 2003; Callaerts-Vegh et al., 2006), whereas mGlu8 knockout mice showed an anxiogenic-like phenotype (Linden et al., 2002; Duvoisin et al., 2005; Robbins et al., 2007). However, in the present study, EPM performance (a prototypic anxiety test for mice) did not discriminate mGlu7- or mGlu8-deficient mice from the other mouse lines. Other authors have also failed to observe increased anxiety in mGlu8 knockout mice (Fendt et al., 2010; Davis et al., 2013), but the lack of EPM alterations in mGlu7 knockout mice was unexpected. Mice in the present study were bred in an SPF facility and transported to the behavioral laboratory at 12 weeks of age, which has been shown to affect EPM behavior (Mineur and Crusio, 2009) and which is different from the other studies that used on-site bred mice. However, mGlu7 knockout mice did display reduced exploratory rearing (in OF and SE), as well as an overall decrease in freezing during the CFC task in agreement with a previous report (Masugi et al., 1999). Freezing to both context and cue are comparable with freezing levels during the shock trial, indicating that mGlu7 knockout mice show an impairment in fear-induced freezing while still able to recall the fear response associated with conditioning context and cue. Masugi et al. (1999) argued that this can be attributed to a lack of mGlu7 receptors in the amygdala.

Cognitive alterations were hypothesized to occur in all 3 mouse strains on the basis of the brain expression patterns of these receptor subtypes (Callaerts-Vegh et al., 2006; Fendt et al., 2010). In agreement with our previous observations (Callaerts-Vegh et al., 2006) and mGlu7's prominent telencephalic distribution (Table 1), mGlu7$^{-/-}$ mice indeed showed impaired MWM learning, which is generally considered to be strongly hippocampus dependent (D'Hooge and Deyn, 2001; Goddyn et al., 2006). Surprisingly, none of the other mouse lines displayed cognitive defects, but mGlu4$^{-/-}$ mice swam considerably faster than the other mice in this task, which could not be reduced to some kind of general restlessness or hyperactivity. Possibly mGlu4$^{-/-}$ mice might be more motivated to reach the platform, since the motivational aspects of learning are indeed expressed by reward approach velocity (Lubbers et al., 2007). Significantly, mGlu4 receptors do occur in nucleus accumbens (Corti et al., 2002), an important area for reward learning and motivation (Robbins and Everitt, 1996). Nucleus accumbens is the interface between limbic and motor systems and translates motivation into action (Mogenson et al., 1980).

To analyze the differences between the studied mouse lines more in detail, the set of behavioral variables was subjected to correlation analysis and DFA. Correlation analyses revealed intra- and inter-task correlations between behavioral measures, whereas DFA, more specifically the stepwise-forward method, indicated that the 3 different knockout groups could be reliably discriminated by some of the behavioral variables. These analyses decisively confirm that the different group III receptors do play distinct behavioral roles. DFA has only been used in a few rodent studies but has been proven successful to distinguish between behavioral profiles of different mouse strains (Leighty et al., 2004; Caeyenberghs et al., 2006). DFA conclusively discriminated between the knockout groups examined here. We used both direct and stepwise-forward methods on the set of behavioral variables (and body weight). Direct entry did not identify any discriminating variable, whereas the stepwise method did (ie, body weight, swimming velocity in MWM, freezing to tone during CFC, and OF_R significantly dissociated the knockout groups).

Various intra-task correlations were found between OF, SE, MWM, and CFC measures, indicating that variables within these tests might measure the same underlying trait. When correlations were calculated per wild-type or knockout group, different correlation patterns were observed. In wild-type mice, strong intra-task correlations were observed for almost each task, whereas in knockout mice, only the SE task showed significant intra-task correlations. In mGlu4$^{-/-}$ mice, velocity in the MWM correlated negatively with path length in the first block of MWM learning, but not with any other activity-related variable (confirms that the altered swimming velocity is not due to general hyperactivity). Conversely, negative correlations between freezing to tone (during CFC) and IR beam crossings (during cage activity and EPM exploration) in mGlu7$^{-/-}$ mice suggests that reduced freezing can be (at least partially) explained by hyperactivity in these animals.

Although several authors already suggested different behavioral functions between group III metabotropic receptors, comparison across the different reports remained methodologically

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hazardous and controversial. Therefore, the present study directly compared knockouts for each group III receptor subtype using a single behavioral test battery and multivariate methods. On the basis of this controlled comparison, we are presently able to conclude that the different group III receptors indeed have quite distinct behavioral functions. By and large, it appears that mGlu7 plays a significant role in spatial learning and in some fear-related behaviors, whereas mGlu4 is most clearly involved in startle and motivational processes. Excepting its influence on body weight, the effect of mGlu8 deletion on behavior appears more subtle than that of the other group III receptors. Importantly, these subtle effects might be due to potential developmental compensatory changes. Raber and Duvoisin (2015) argue that other receptors (eg, mGlu4) might compensate for the lack of this receptor subtype. This plausible compensation for receptor subtype-specific deficits is not only limited to mGlu8 receptor but is applicable to all 3 subtypes. To fully understand the differential role of group III receptor subtypes, studies using either conditional single- or double knockouts or RNA interference techniques (O'Connor et al 2013) might provide an alternative approach.

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Statement of Interest

None.

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